Application for

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of

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for

METHODS FOR COVALENTLY ATTACHING NUCLEIC ACIDS TO SUBSTRATES

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the field of means of attaching nucleic acids, peptides, polypeptides, and other biomolecules to various surfaces, including microarrays, silicon wafers, glass beads, glass slides, plastics, membranes, metals, or other irregular surfaces and solutions containing particles with similar surfaces.

Description of the Prior Art

Accompanying the development of several high-throughput technologies that make it possible to sequence complex genomes is the challenge to not only identify genes, but to understand the function and expression of those genes. cDNA microarray technology allows for the high-throughput measurement of expression patterns of thousands of genes simultaneously. Microarrays utilize a high-speed precision robot, ink-jet printing heads, or other technologies to affix thousands of DNA samples onto a solid support (a glass slide, chip or nylon membrane). This allows high spot densities to be obtained, thus increasing the number of samples that can be analyzed at once. The slides are simultaneously probed with fluorescently-labeled cDNAs generated from mRNA isolated from cells or tissues in two different physiological, developmental or disease states (*Science*, 278, 680-686; *Nature Genet.*, 14, 457-460, 1996; *Science*, 278, 680-686, 1997; *Proc. Natl. Acad. Sci., USA*, 94, 2150-2155, 1997; *Curr. Opin. Gen. Dev.*, 7, 771-776, 1997; and *Nature Biotechnol.*, 15, 1359-1367, 1997). Each population of cDNA is labeled with a different fluorescent dye, allowing direct comparisons on a single array. The relative intensities of the two fluorescent dyes within a spot correspond to the relative expression levels of the genes, reflected by the two RNA populations used to make the labeled cDNAs.

Thus, the major steps of DNA microarray technology include the manufacture of microarrays, the fluorescent labeling of cDNA probes, hybridization of the probes to the immobilized target DNA, and the subsequent analysis of the hybridization results.

The use of microarrays, therefore, allows the expression of thousands of genes to be assayed in a single experiment. DNA microarray technology was initially developed to detect global patterns of gene expression. However, it has many other potential important applications including identification of complex genetic diseases (*Nature Genet.*, 18, 225-230, 1998), mutation/polymorphism detection (*Science*, 280, 1077-1082, 1998), and drug discovery and toxicology studies (*Nature Genet.*, 1 (Suppl), 48-50, 1999). Recently, tissue microarrays were used for the molecular profiling of tumor specimens (*Nature Med.* 4, 844-847, 1998). Also, protein microarrays were developed for high-throughput analysis of protein function (*Science*, 289, 1760-1763).

Current technology leads to a problem of reusability. There is a need for increased uniformity and strength of attachment, which would result in more uniform fluorescent signals and decreased DNA loss during the course of experiments. Also, if more stringent wash conditions were feasible, this could reduce background and thus allow for greater sensitivity. There is thus a need for increased reproducibility and consistency of results, and enhanced stability that allows the reuse of microarrays. None of the existing technologies provide a means to fulfill these needs.

SUMMARY OF THE INVENTION

It is therefore an object of this invention to provide a means to attach nucleic acids, peptides, polypeptides or other biomolecules to surfaces such as glass, silicon, and derivative substrate.

It is a further object of the present invention to provide a microarray.

It is yet another object to provide a method for forming a microarray.

It is yet another object to provide a microarray that utilizes a glass or plastic surface.

It is yet another object to provide a microarray that utilizes a noble metal surface.

It is yet another object to provide a microarray that utilizes a metal oxide surface.

It is yet another object to provide a washable microarray that allows for repeated use.

In all of the above embodiments, it is an object to provide a microarray which covalently binds biomolecules to a surface.

According to a first broad aspect of the present invention, there is provided a microarray comprising a substrate with a siloxy diazotized surface, and at least one biomolecule covalently bound to the siloxy diazotized surface.

According to a second broad aspect of the present invention, there is provided a siloxy diazotized surface.

According to a third broad aspect of the invention, there is provided a method for forming a microarray comprising treating an oxidized surface with a siloxy amine to form a siloxy amine treated surface, treating the siloxy amine surface with a diazotizing agent to form a siloxy diazotized surface, and contacting the siloxy diazotized surface with at least one biomolecule to form a microarray in which at least one biomolecule is covalently bound to the siloxy diazotized surface.

According to a fourth broad aspect of the invention, there is provided a method for forming a siloxy diazotized surface comprising treating an oxidized surface with a siloxy amine to form a siloxy amine treated surface; and treating the siloxy amine treated surface with a diazotizing agent to form a siloxy diazotized surface.

According to a fifth broad aspect of the present invention, there is provided a microarray comprising a substrate with a thiolate diazotized surface, and at least one biomolecule covalently bound to the thiolate diazotized surface.

According to a sixth broad aspect of the present invention, there is provided a thiolate diazotized surface.

According to a seventh broad aspect of the invention, there is provided a method for forming a microarray comprising treating a noble metal surface with a thiolate amine to form a thiolate amine treated surface, treating the thiolate amine surface with a diazotizing agent to form a thiolate diazotized surface, and contacting the thiolate diazotized surface with at least one biomolecule to form a microarray in which at least one biomolecule is covalently bound to the thiolate diazotized surface.

According to a eighth broad aspect of the invention, there is provided a method for forming a thiolate diazotized surface comprising treating a noble metal surface with a thiolate amine to form a thiolate amine treated surface; and treating the thiolate amine treated surface with a diazotizing agent to form a thiolate diazotized surface.

According to a ninth broad aspect of the invention, there is provided a method for using a microarray comprising the steps of contacting the microarray with a plurality of first free biomolecules to hybridize or attach to at least a portion of the plurality of first free biomolecules to bound biomolecules of the microarray; and removing the first free biomolecules from the microarray without removing the bound biomolecules from the microarray.

Other objects and features of the present invention will be apparent from the following detailed description of the preferred embodiment.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be described in conjunction with the accompanying drawings, in which:

FIG. 1 is a schematic diagram of the attachment of a tethered moiety to a solid substrate.

FIG. 2 is a schematic diagram of a primary aromatic amine (ATMS) diazotization method for covalent immobilization of double-stranded target DNA on a glass surface and subsequent hybridization of probe DNA, constructed in accordance with a preferred embodiment of the invention;

FIG. 3 is a schematic diagram of a primary aromatic amine diazotization method for covalent immobilization of single-stranded target DNA on a noble metal surface and subsequent hybridization of probe DNA, constructed in accordance with a preferred embodiment of the invention;

FIG. 4 is an image comparison of Cy3-labeled *am* DNA spotted onto ATMS/diazotized, polylysine-coated and TeleChem (silyated) glass microscope slides;

FIG. 5 is a graphical plot of intensity of Cy3-labeled *am* DNA versus spotting on ATMS/diazotized (ATMS), polylysine (PLY) and TeleChem (TEL) surfaces;

FIG. 6a is an image of an ATMS/diazotized glass slide arrayed with Cy5-labeled *am* DNA in dilutions of 1:1, 1:2, 1:5, 1:10, 1:25, 1:50 and 1:100 in 3X SSC (3 replicas of all dilutions);

FIG. 6b is a graphical plot of average intensity of spots versus each dilution in FIG. 6a;

FIG. 7 is an image comparison of hybridization of Cy5-labeled *am* probe to *am* target DNA on ATMS/diazotized, Corning and polylysine glass microscope slides;

FIG. 8a is an image of scanned spots of the hybridization of Cy5-labeled *am* DNA probe to increasing amounts of target DNA;

FIG. 8b is a graphical plot of average intensity versus each scanned spot of FIG. 8a;

FIG. 9a is an image of scanned spots of subsequent hybridizations that show the effect of stripping on re-hybridization of Cy5-labeled *am* probe to varying amounts of target DNA immobilized on an ATMS/diazotized surface;

FIG. 9b is a graphical plot of average intensity versus each scanned spot of FIG. 9a; and

FIG. 10 is a graphical plot of time versus reflectance for SPR data collected for the hybridization of DNA to the thiolated/diazotized gold surface.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

It is advantageous to define several terms before describing the invention. It should be appreciated that the following definitions are used throughout this application.

Definitions

Where the definition of terms departs from the commonly used meaning of the term, applicant intends to utilize the definitions provided below, unless specifically indicated.

For the purposes of this invention, the term "microarray" refers to a device that employs the attachment of biomolecules to a substrate.

For the purposes of this invention, the term "siloxy diazotized surface" refers to a surface, such as shown by element 204 in FIG. 2, which contains a diazonium ion attached through a siloxy group to a glass substrate.

For the purposes of this invention, the term "siloxy diazotized glass surface" refers to a surface that contains a diazonium ion attached to a phenyl ring para to an aromatic attached trimethoxysilane bound to the glass substrate.

For the purposes of this invention, the term "oxidized surface" refers to an oxygen containing surface (e.g. of a metal oxide or one that has been treated with an oxidizing agent) such that it is reactive to a siloxy amine.

For the purposes of this invention, the term "siloxy amine" refers to an aromatic amine, $-NH_2$, functional group attached to a siloxy group.

For the purposes of this invention, the term "siloxy amine treated surface" refers to a surface coated with siloxy amines.

For the purposes of this invention, the term "glass surface" or "glass substrate" refers to glass used in the microarray that attaches to ATMS with silanol groups.

For the purposes of this invention, the term "thiolate diazotized surface" refers to a surface, such as shown by element 304 in FIG. 3, which contains a diazonium ion attached through a thiolate group to a noble metal substrate.

For the purposes of this invention, the term "thiolate diazotized noble metal surface" refers to a surface that contains a diazonium ion attached to a phenyl ring para to an aromatic attached thiolate bound to the noble metal substrate.

For the purposes of this invention, the term "thiol amine" refers to an amine, $-NH_2$, functional group attached to a thiolate group.

For the purposes of this invention, the term "thiolate amine treated surface" refers to a surface coated with thiolate amines.

For the purposes of this invention, the term "noble metal surface" or "noble metal substrate" refers to noble metal used in the microarray that attaches to 4-aminothiophene with thiolate groups.

For the purposes of this invention, the term "diazotized" refers to the attachment of a diazonium ion to the phenyl ring in the microarray.

For the purposes of this invention, the term "diazotizing agent" refers to a reagent containing nitrous acid. FIG. 2 and 3 illustrate use of $NaNO_2$ and HCl as the diazotizing agent.

For the purposes of this invention, the term "siloxy amine" refers to a primary aromatic amine such as ATMS or similar compound that attaches to the silanol groups of the glass surface of the microarray.

For the purposes of this invention, the term "thiolate amine" refers to a primary aromatic amine such as 4-aminothiophene or similar compound that attaches to the noble metal surface of the microarray.

For the purposes of this invention, the term "ATMS" refers to p-aminophenyl trimethoxysilane, which attaches to the silanol or other groups on the glass surface.

For the purposes of this invention, the term "biomolecule" refers to nucleic acids, modified RNA, aptamers, proteins, polypeptides, including antibodies and fragments thereof, and similar or related chemical compounds.

For the purposes of this invention, the term "free biomolecule" refers to a biomolecule that is not covalently bound to a substrate or hybridized with or attached to the bound biomolecules.

For the purposes of this invention, the term "bound biomolecules" refers to biomolecules covalently bound to a substrate and that are hybridizable with or can attach to free biomolecules.

For the purposes of this invention, "hybridization" refers to the chemical interaction and attachment of free nucleic acids to the nucleic acids attached to the surface of the microarray.

For the purposes of this invention, the term "stripping" refers to the removal of a hybridized or attached biomolecule on a microarray.

For the purposes of this invention, the term "silanol group" refers to a hydroxy group bound to silicon.

For the purposes of this invention, the term "surface" refers to silicon wafers, glass beads, glass slides, plastics, membranes, metals, or other regular or irregular surfaces and solutions containing particles with similar surfaces.

For the purposes of this invention, the term "aptamers" refers to non-immunogenic compounds that bind to targets with high affinity and specificity.

Description

The present invention relates broadly to the attachment of biomolecules, such as nucleic acids and proteins, to various surfaces, which include microarrays, silicon wafers, glass beads, glass slides, plastics, membranes, metals, or other irregular surfaces and solutions containing particles with similar surfaces. While the chemistry described hereinafter applies to the attachment of biomolecules to any of these surfaces, the remaining discussion will focus on the preferred embodiment of attaching biomolecules to a glass slide to create a microarray.

The present invention produces a chemically reactive surface on substrates to which DNA may covalently bind. Subsequently, DNA can hybridize to fluorescently-labeled cDNA probes in a reproducible, consistent, uniform and stable manner. The covalent binding of DNA to solid substrates should increase the uniformity of attachment, and result in more uniform fluorescent signals and decreased DNA loss during the course of experiments. Covalently bound DNA should also permit the use of more stringent wash conditions, which would reduce background and thus allow for greater sensitivity. The binding chemistry described here provides increased reproducibility and consistency of results, and enhanced stability that allows the reuse of microarrays. In addition, this chemistry does not require prior synthetic modification of DNA. This approach is also applicable to the attachment of other nucleic acids, modified RNA, aptamers, proteins, polypeptides, including antibodies and fragments thereof, and similar or related chemical compounds. Furthermore, standard procedures involving DNA, RNA, proteins and/or other biomolecules, such as hybridizations, specific ligand-receptor or other protein binding, or polymerase chain reaction, may be performed on the microarray, other than the examples provided herein, for fluorescent detection of cDNA interactions.

There are two current predominant methods for producing DNA microarrays. The first was developed by Stephen Fodor and colleagues in the early 1990's (*Science*, 251, 767-773, 1991), and it is often referred to as the Affymetrix method (Affymetrix, Inc., Santa Clara, CA). In this process, DNA oligonucleotides are synthesized directly onto a microarray or DNA chip using photo-labile protecting groups and masks to direct the selective addition of nucleotides.

In the second common method for producing microarrays, pre-existing DNA fragments are spotted onto a glass or membrane support using a precision robot (*Science,* 270, 467-470, 1995).

Both of these systems commonly permit only single usage of the DNA arrays. Reusability of microarrays would eliminate the variance between arrays, which are often presumed identical. The variance ultimately affects the experimental reliability of microarray-based analyses. (Lack of reusability is, ostensibly, due to the quality of the glass substrate.) The surface chemistry of the glass substrate is a major determinant of the stability of DNA attachment throughout the hybridization and washing steps.

The most popular substrates for spotting DNA are currently polylysine and aminosilane coated glass slides (*Nature Gen.* (Supplement), 21, 25-32, 1999). These slides are available commercially, suitable for miniaturizing array dimensions and fluorescence detection, inexpensive, and ready-to-use. The disadvantages of these slides include: 1) the spots are not uniform, limiting the accuracy of quantitative detection, and 2) the high background commonly seen decreases the sensitivity of detection (*Nucleic Acids Res.*, 27, 1970-1977, 1999).

As discussed earlier, the lack of reusability presents a big problem, but a substrate that allows covalent attachment of nucleic acids could solve the problem of reusability. The derivatization method of the present invention to covalently immobilize DNA onto a glass substrate for subsequent hybridization with DNA probes can solve problems with the prior art. The present invention produces a chemically reactive surface on a substrate to which DNA will covalently bind and, subsequently, hybridize with fluorescently-labeled cDNA probes in a reproducible, consistent, uniform and stable manner. The present invention involves a method for covalently linking DNA and other biomolecules to a substrate in a manner that preserves the ability of the immobilized biomolecule to hybridize with complementary sequences or attach to molecules with binding affinity.

FIG. 2 illustrates a schematic of an ATMS/diazotization method for covalent immobilization of double-stranded target DNA on a solid surface and subsequent hybridization of probe DNA, in accordance with a preferred embodiment of the invention. FIG. 2 shows that a substrate 200 is washed and oxidized with piranha solution and functionalized with p-aminophenyl trimethoxysilane (ATMS) to form the siloxy amine treated surface 202. FIG. 2 then illustrates that siloxy amine treated surface 202 is treated with a solution containing hydrochloric acid (HCl) and sodium nitrite ($NaNO_2$) to form the diazotized surface 204. FIG. 2 next demonstrates that diazotized surface 204 is exposed to double stranded DNA to microarray or spot the ssDNA onto the diazotized glass surface to form a microarray 206. Fig. 2 then illustrates that the dsDNA attached to the diazotized glass surface is baked to yield ssDNA attached to the diazotized glass surface of microarray 208. Lastly, FIG. 2 shows the hybridization of free single-stranded DNA to microarray 208 to yield microarray 210.

FIG. 3 illustrates a schematic of a 4-aminothiophene/diazotization method for covalent immobilization of single-stranded target DNA on a solid surface and subsequent hybridization of probe DNA, in accordance with a preferred embodiment of the invention. FIG. 3 shows that a substrate 300 is functionalized with 4-aminothiophene to form the thiolate amine treated surface 302. FIG. 3 then illustrates that the thiolate amine treated surface 302 is treated with a solution containing hydrochloric acid (HCl) and sodium nitrite ($NaNO_2$) to form the diazotized surface 304. FIG. 3 next demonstrates that diazotized surface 304 is exposed to single-stranded DNA to microarray or spot the ssDNA onto the diazotized glass surface to form a microarray 306. Lastly, FIG. 3 shows the hybridization of free single-stranded DNA to microarray 306 to yield microarray 308.

Though Figures 2 and 3 demonstrate double-stranded DNA attached to an oxidized glass surface and single-stranded DNA attached to a noble metal surface, respectively, both single- and double-stranded DNA, as well as other biomolecules, can be covalently attached to either glass or noble metal surfaces.

The present invention will now be described by way of example.

EXAMPLE 1

Attachment of Tethered Moiety to a Solid Substrate

While the chemistry described in the previous examples applied to the attachment of biomolecules, in particular DNA, to glass or plastic surfaces, it is important to note once more that it can be applied to the attachment of biomolecules to a host of surfaces, including microarrays, silicon wafers, glass beads, glass slides, plastics, membranes, metals, or other irregular surfaces and solutions containing particles with similar surfaces. This example describes the attachment of an amine moiety to a solid surface.

The surface chemistry of the present invention description is based on the diazotization of aromatic amine surfaces. Suitability of the solid surface requires the presence of a suitable tether that is used to attach aromatic amine moieties. Examples of tether moieties include, but are not limited to trialkoxy or trichloro silanes for immobilization to oxide surfaces (e.g., glass) or thiols for immobilization on noble metal (e.g. Au, Ag, Cu) surfaces. FIG. 1 shows a schematic of the attachment of a tethered moiety to a solid substrate. FIG. 1 shows a tether group that attaches to a reactive site on the solid substrate. Once the tether group reacts with the solid substrate, FIG. 1 shows that the amine group is available for reaction with $NaNO_2$ and HCl to form the diazonium ion as demonstrated in FIG. 2 and FIG. 3 and described later in Examples 2 and 6. The solid substrate with the amine group attached can now undergo the chemistry involved in the diazotization attachment discussed below.

EXAMPLE 2

Method for Covalently Linking DNA to Glass, Plastic, and Other Substrates

The present invention can utilize a variety of substrates, as described in Example 1. With the exception of noble metal substrates (as described later in Example 6)) and aromatic polyamide substrates (which may still contain aromatic amine monomers following polymerization and, as such, can possibly undergo diazotization directly), primary aromatic amines are generally introduced to oxidized substrates, e.g., glass and plastic, by silanization. Hydroxyl groups are added to these substrates by surface oxidation reactions. For example, glass and silicon can be treated with an oxidizing agent such as piranha solution. Polymers, e.g. plastics, can be oxidized by a variety of oxidation techniques including the use of corona discharge, ozone, oxygen plasma, hydrogen peroxide, nitrous acid, alkaline hypochloride, UV irradiation, oxidizing flame, and chromic acid (*Polymer Science and Technology*, R. Ebewele, 2000; *Journal of Adhesion*, 43 (#1-2), 139-155, 1993; *Journal of Micromech. Microeng.*, 9, 211-217, 1999; *.Appl. Phys. Let.*, 75(#17), 2557-2559, 1999; *Langmuir*, 14, 5586-5593, 1998; *Journal of Adhesion Sci. Tech.*, 11(#7), 995-1009, 1995; *Syn. Metals*, 60(#2), 93-96, 1993). Gutowski *et al.* used the process of corona discharge followed by application of organo-functional silane to accomplish the silanization of polyethylene. The incorporation of surface hydroxyl groups onto the polymer

surface enables organo-silane to create the hydrogen or covalent bonds with the oxidized polymer surface (*Journal of Adhesion*, 43 (#1-2), 139-155, 1993). Whitesides *et al.* have successfully accomplished the oxidation of different surfaces (polymer, glass, silicon, silicon oxide) through oxygen plasma (*Journal of Micromech. Microeng.*, 9, 211-217, 1999;.*Appl. Phys. Let.*, 75(#17), 2557-2559, 1999). Fadeev and McCarthy's research shows that a 3-aminopropyltrialkoxysilanes modified polyethylene terephthalate (PET) surface followed by hydrolysis is reactive to organosilanes and should react with the versatility of oxidized silicon wafers (*Langmuir*, 14, 5586-5593, 1998). Recent research also shows that silane can be used in modifying clay and ceramic (*Appl. Clay Sci.*, 15(#1-2), 51-65, 1999; *Ceramic Int.*, 21(#3), 181-186, 1995). Thus, based on the theory and results of recent researches, hydroxyl group terminated surfaces can include oxidized glass, oxidized silica surface, and oxidized polymer. Clay and ceramic can be modified by silane. Glass, silicon wafer, polymer, clay and ceramic would thus all be suitable substrates for the present invention after the pre-treatment of oxidization.

The present invention involves a chemical process for covalently linking DNA to a substrate in a manner that preserves the ability of the immobilized nucleic acid to hybridize to complementary sequences. A schematic of the overall process is shown in FIG. 2. In brief, glass substrates were first cleaned and oxidized with piranha solution and then functionalized with p-aminophenyl trimethoxysilane (ATMS).

Just before reaction with nucleic acids (referred to herein as target DNA), ATMS-reacted surfaces were converted to the diazobenzyl form by treatment with a solution containing HCl and $NaNO_2$. Primary aromatic amines react with nitrous acid to yield diazonium salts. In this step, an electrophilic attack by ^{+}NO causes displacement of the H^{+} at the nitrogen (*Organic Chemistry*. (Third ed.), 1973):

Because diazonium salts are unstable, this and all subsequent steps of this process, inclusive of nucleic acid spotting, were done at 4° C. After 30 minutes, the ATMS-treated substrates were washed successively with ice-cold sodium acetate buffer, double-distilled H_2O, and 100% ethanol. A gentle acidic buffer keeps the diazonium salts active (*Organic Chemistry*. (Third ed.), 1973) on the glass surface. DNA was then spotted or microarrayed onto the diazotized glass surfaces, and air-dried at room temperature for 1-2 hours. The DNA reacts to form a covalent bond with the azo-terminus of the diazotized surface (*Organic Chemistry*. (Third ed.), 1973). In order to neutralize unreacted diazonium groups and to reduce nonspecific binding of the probe to the slide, the surfaces were either immersed in 1% glycine solution or prehybridized in a buffer containing 50% formamide, 5X SSC, 0.1% SDS and 1% BSA.

Diazonium salts can undergo a reaction referred to as "coupling," in which certain aromatic compounds (such as the bases in DNA or other biomolecules) covalently bind to the positively-charged nitrogen of the diazonium group (*Organic Chemistry*. (Third ed.), 1973). In the reaction between the diazonium salt on the glass surface and the DNA molecule, the aromatic rings of the DNA bases undergo attack by the diazonium ion. Because the diazonium ion is very weakly electrophilic, the aromatic ring must contain a powerful

electron-releasing group, i.e., -OH, -NR_2, -NHR, or -NH_2. Covalent binding usually occurs *para* to the activating (electron-releasing) group (*Organic Chemistry*. (Third ed.), 1973). In the structure of the four bases of DNA, adenine, cytosine and guanine all contain an aromatic-NH_2 group, which may undergo the coupling reaction with the diazonium ion. However, there is no strong electron-releasing group present in thymine. We thus assume that the covalent binding would occur between adenine, cytosine or guanine bases of the DNA and the diazonium salt. The thymine residue is covalently bound to the positively charged amine groups on the surface of the substrate by ultraviolet irradiation. Similar reactions occur with other biomolecules, in that other biomolecules containing an aromatic ring with a powerful electron-releasing group may also undergo the coupling with the diazonium ion.

The layer formation of ATMS onto glass substrates in this example was done as follows. Glass or silicon substrates requiring a pre-oxidation step were cleaned by immersion into piranha solution (70/30 v/v sulfuric acid and 30% hydrogen peroxide) for 30 minutes followed by washing in deionized water (*Langmuir*, 12, 4621-4624, 1996). Cleaned substrates were coated with ATMS by immersing the substrates in 1 mM solution of p-aminophenyl trimethoxysilane in ethanol for 30 minutes. The substrates were then rinsed in ethanol and dried in a stream of N_2. This procedure resulted in the formation of an amine-terminated layer on the substrate. Formation of silane layers was confirmed by X-ray photoelectron spectroscopy. The thickness of the ATMS layers was estimated by ellipsometry of monolayers formed on surface-oxidized Si wafers (*Langmuir*, 12, 4621-4624, 1996). The ellipsometric thickness of this ATMS layer was 4.9 ± 0.2 Å.

The diazotation of ATMS-treated surfaces in this example was done as follows. Just before reaction with nucleic acids, ATMS-reacted surfaces were converted to the diazobenzyl form by treatment with a solution containing 120 ml of water, 240 ml of 400 mM HCl, and 9.6 ml of freshly prepared solution of 200 mM $NaNO_2$ for 30 minutes at 4 °C (14). After 30 minutes, the ATMS-treated surfaces were washed 3 times, each for 3 minutes, with ice-cold sodium acetate buffer (50 mM, pH 4.7) followed by washing with ice-cold deionized water and then ethanol 2 times each (5 minute washes). The diazotized surfaces were air-dried and gently blotted with Kimwipes® (Kimberly-Clark, GA) while maintaining their temperature at 4 °C.

The preceding method of layer formation of siloxy amines and diazotization of siloxy amine treated surfaces can be applied to other oxidized substrates such as, plastics, clay, ceramics, and membranes. Though the diazonium salts on the glass surface are unstable and, thus, remain optimally active at a temperature of 4 degrees Centigrade, the diazotized surface will remain significantly, though less, active for hours at room temperature. Correspondingly, while covalent attachment of biomolecules is optimal at 4 degrees Centigrade, covalent attachment will occur at room temperature for hours following diazotization of the surface. In certain cases such use of higher temperatures may be more practical and the resulting attachment is acceptable. Additionally, the reaction will work, at least partially, for incubation times less than 30 minutes with acceptable but less than optimal results. It may also be possible to use common salts, such as ZnCl, as stabilizers of the diazonium ions, which may ameliorate the decreased attachment and annealing performance at temperatures above 4 C. Such procedures are also anticipated by this invention.

EXAMPLE 3

Attachment of Cy Dye-labeled DNA to ATMS/diazotized Glass Substrates

Preparation of DNA and labeling of probes was done as follows:

Genomic DNA from the filamentous fungus *Neurospora crassa* was isolated as described (*Neurospora Newsletter*, 29, 27-28, 1982) from the *N. crassa* wild-type ORS*a* strain (FGSC strain 2490; Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, KS). *N. crassa am* DNA, corresponding to the glutamate dehydrogenase gene (*Gene*, 20, 387-396, 1982), was a 2 kbp *Eco*RI-*Bam*HI fragment from pBJ011 (21), gel purified with the QIAEX® II Gel Extraction System and QIAquick Gel Extraction Kit (Qiagen, Inc, CA.). These DNAs were resuspended in 10 µl ddH_2O or 4X SSC and immobilized on diazotized glass surfaces at each of the following concentrations per microliter: 100 ng ORS*a*; 100 ng ORS*a* plus 0.05 ng *am*; 100 ng ORS*a* plus 0.5 ng *am*; 100 ng ORS*a* plus 5.0 ng *am*; and 100 ng *am* (where ORS*a* refers to DNA from the entire genome of *N. crassa*). There is one gene-equivalent of the *am* gene per one genome equivalent.

The *am* DNA probes were fluorescently labeled with Cy3™-dCTP or Cy5™-dCTP (Amersham Pharmacia Biotech, NJ) using a Nick Translation Kit for radioactive and non-radioactive probe preparation (Amersham Pharmacia Biotech, NJ). After labeling, the probes were precipitated with 1 volume of 4 M ammonium acetate and 5 volumes 100% ethanol and resuspended in 6-8 µl of sterile ddH_2O.

The attachment of the DNA onto the siloxy diazotized surface was done as follows. The experimental DNAs were printed onto the ice-cold diazotized surfaces (manually or using a micro spotting device). After air-drying for 1-2 hours at room temperature, the diazotized slides were immersed in 1% glycine solution (pH 7.2) twice, each time for 5 minutes, rinsed with ddH_2O to remove the residue of glycine, and air-dried. The printed slides were stored in the dark at room temperature until used for scanning.

Results indicate that our immobilization method produces uniform spots that immobilize equal or greater amounts of DNA than do current commercially available immobilization techniques (e.g. polylysine-based methods (*Science,* 270, 467-470, 1995)). FIG. 4 shows images that were captured from double-stranded Cy3-labeled *am* DNA spotted onto ATMS/diazotized slides, polylysine-coated slides (Sigma, MO), and silylated slides (TeleChem International, Inc., CA). Cy3-labeled DNA was manually spotted with a p10 micro Pipetman (Rainin Instrument Company, Emeryville, CA) onto diazotized, polylysine, and TeleChem glass slides in dilutions of 1:10, 1:100, and 1:1000 using both H_2O and 3X SSC as diluents. The protocol used for processing the TeleChem slides was that suggested by the manufacturer (http://arrayit.com/Products/SuperMicroarray_Substrates/supermicroarray_substrates.html#CP). Processing of polylysine slides was as previously described (*Nature Genet.*, 14, 457-460, 1996). The spots on the diazotized slides were more homogeneous and uniform than those on the polylysine and TeleChem slides, and background fluorescence was significantly lower.

The individual, manually printed, spots were visualized with a Nikon Diaplot laser-confocal microscope (Nikon, Inc., NY) equipped with a Bio-Rad MRC-600 scanning head (Bio-Rad, CA).

FIG. 5 is a graphical plot of intensity of Cy3-labeled *am* DNA versus spotting on ATMS/diazotized (ATMS), polylysine (PLY) and TeleChem (TEL) surfaces. FIG. 5 shows that the results with diazotized slides were quantifiable and reflected the serial dilutions used, in contrast to the spots on the polylysine and TeleChem slides. FIG. 6a shows images captured from Cy5-labeled *am* DNA microarrayed onto ATMS/diazotized glass (with the Liedtke Arrayer, University of New Mexico, Department of Mechanical Engineering). The images of FIG. 6a show Cy5-labeled DNA was microarrayed onto a diazotized glass slide in dilutions between 1:1 and 1:100. FIG. 6b is a graphical plot of average intensity of spots versus each dilution in FIG. 6a.

The array was scanned using the ScanArray 3000 (GSI Lumonics, CA), and the spots were automatically pseudo-colored by the software program. As with manual spotting, uniform and homogeneous spots were imaged that reflected the serial dilutions. The most intense spots (left-most column) were seen for the most concentrated sample, with the intensity of each column of spots decreasing as the sample became more dilute.

Also, in an attempt to evaluate and disrupt the covalent attachment, the ATMS/diazotized slides spotted with the Cy3-labeled DNA were processed through a stripping procedure. The slides were immersed in stripping buffer (2.5 mM Na_2HPO_4, 0.1% SDS) at 95° C for 30 seconds, three to four times (*Nucleic Acids Res.*, 27, 1970-1977). They were then rinsed in ddH_2O, air-dried, and stored in the dark at room temperature. The Cy3-labeled DNA remained affixed to the diazotized surface.

EXAMPLE 4

Hybridization of DNA Array

Unlabeled target DNA was immobilized on ATMS/diazotized, polylysine and Corning amino-silane coated slides (CMT-GAPS™, Corning, Inc.). Cy5-labeled *am* DNA was hybridized to increasing amounts of target DNA in the spotted DNA solutions: 20 ng genomic (ORS*a*) DNA; 0.01 ng *am* DNA in 20 ng ORS*a*; 0.1 ng *am* in 20 ng ORS*a*; 1 ng *am* in 20 ng ORS*a*; and 20 ng *am* DNA. FIG. 5 is an image comparison of hybridization of Cy5-labeled *am* probe to *am* target DNA on ATMS/diazotized, Corning and polylysine glass microscope slides.

The hybridization with ATMS/diazotized slides and Corning slides was done as follows. The printed slides were UV-crosslinked at 90 mJ using a Stratalinker™ (Stratagene, CA) and baked at 80° C for two hours. The slides were stored in the dark at room temperature until used for hybridization. Prior to hybridization, the printed arrays were placed into a Coplin jar and prehybridized for 45 minutes at 42°C in a buffer containing 50% formamide, 5X SSC, 0.1% SDS and 1% BSA. After prehybridization, the slides were washed in room temperature ddH_2O and isopropanol and then air-dried. The Cy5-labeled probes were denatured for 5 minutes in boiling water, then combined with equal volumes of 2X hybridization buffer (50% formamide, 10X SSC, 0.2% SDS) preheated to 42° C. The hybridization mixture was applied onto the array, and covered with a glass cover slip. The array was placed in a humid ArrayIt™ Hybridization Cassette (TeleChem International, Inc., CA) and submerged in a 42° C water bath for 14-16 hours. After hybridization, the array was washed at room temperature in 1X SSC, 0.1% SDS for 5 minutes, 0.1X SSC, 0.1% SDS for 5 minutes, and 0.1 X SSC for 5 minutes. The array was given a final, brief rinse in ddH_2O and air-dried.

The hybridization with the polylysine slides was done as follows. The Cy5-labeled probes were suspended in 12-15 µl of 4X SSC plus 0.2-0.3 µl 10% SDS. The hybridization mixture was applied onto the array, and covered with a glass cover slip. The array was placed in a humid ArrayIt™ Hybridization Cassette (TeleChem International, Inc., CA) and submerged in a 65° C water bath for 14-16 hours. After hybridization, the array was washed at room temperature by gently plunging up and down in 1) 1X SSC, 0.03% SDS; 2) 0.2X SSC, and 3) 0.05 X SSC. The array was given a final, brief rinse in ddH_2O and air-dried.

The arrays were visualized with the confocal, laser scanner, ScanArray 3000, produced by GSI Lumonics (CA). The hybridized arrays were processed, using Image Tool image processing program, Version 2.0 (University of Texas Health Science Center at San Antonio, TX), and Microsoft Excel (Microsoft Inc., WA). The steps included: 1) identifying each spot location; 2) determining the spot intensity and the fluorescence background; and 3) calculating the background-subtracted intensities of each spot.

Hybridization was detected with even the lowest amount of *am* DNA immobilized on the ATMS/diazotized slide. On the Corning slide, hybridization only with those spots corresponding to the two highest amounts of target DNA (20 ng *am* and 1 ng *am* in 20 ng ORS*a*) was detectable. Furthermore, on the polylysine slide, only the spot corresponding to the highest amount of target DNA (pure *am* DNA) was detectable, and the intensity of this spot was much lower than that of the corresponding spots on ATMS/diazotized or Corning slides (not shown).

FIG. 7 is an image comparison of hybridization of Cy5-labeled *am* probe to *am* target DNA on ATMS/diazotized, Corning and polylysine glass microscope slides. It shows that the spots on the ATMS/diazotized slide were very uniform and homogeneous, and the background fluorescence was extremely low. The spots on the Corning slide were also homogenous, like those on the ATMS/diazotized slide; however, they had high background and "comet tails" streaked from the detectable spots. FIG. 7 also demonstrates that the spots on the polylysine slide lacked clarity due to high background, low intensity and "comet tails" and "shadows".

FIG. 8a is an image of scanned spots of the hybridization of Cy5-labeled *am* DNA probe to increasing amounts of target DNA and FIG. 8b is a graphical plot of average intensity versus each scanned spot of FIG. 8a. FIGS. 8a and 8b show that the ATMS/diazotized surface chemistry facilitated accurate detection of very low proportions of specific target DNA upon hybridization with a Cy5-labeled probe; even the lowest amount of *am* DNA, corresponding to about one-thousandth of the DNA in the spot, was detectable.

High background and the streaking of spots by "comet tails", as seen with the Corning and polylysine slides, have limited the accuracy and reproducibility of microarray analyses. However, with the ATMS/diazotized surface chemistry, hybridization of a probe corresponding to a single target gene allowed reproducible detection of the target sequence within genomic DNA, even when the target gene represented approximately 1/2000 of the genome (and therefore a small fraction of the DNA immobilized on the slide).

EXAMPLE 5

Stripping and Reuse of Arrayed/Diazotized Slides

After an initial hybridization with a fluorescently-labeled probe, the arrayed diazotized slides were stripped and re-hybridized with a Cy5-labeled *am* DNA probe four successive times. Stripping resulted in the removal of essentially all signal (not shown). FIG. 9a is an image of scanned spots of subsequent hybridizations showing the effect of stripping on re-hybridization of Cy5-labeled *am* probe to varying amounts of target DNA immobilized on the ATMS/diazotized surface (where 1 is the initial hybridization, and 2-5 are sequential hybridizations after repeated stripping). FIG. 9b is a graphical plot of average intensity versus each scanned spot of FIG. 9a. FIGS. 9a and 9b show that while the intensity of signals lessened with each successive hybridization, the spots on the ATMS/diazotized slide clearly remained detectable and proportionately accurate, reflecting the amounts of specific target DNA immobilized. The reusability of ATMS/diazotized slides makes this chemistry a significant contribution to the field of DNA microarray technology.

EXAMPLE 6

Attachment of Nucleic Acids to a Gold Substrate

In an extension of Example 1, a thiol tether can be used to attach to a gold solid substrate. FIG. 10 shows that a thiolate groups can attach to a gold substrate. 30 Å of chromium and 560 Å of gold were electro-evaporated on a glass slide pre-cleaned with piranha solution. The gold-coated substrate was immersed in a 1 mM solution of 4-aminothiophene in ethanol for 12 hours (or a 10 mM solution for 1 hour). The layer of amino terminated thiolate was rinsed with water and ethanol, and then dried with a stream of N_2. The aminothiophene-treated surface was diazotized as described in example 2 and shown in FIG. 3, spotted with single-stranded *am* DNA, air-dried for 1 ~ 2 hours, then washed with 1% glycine.

To substantiate that hybridization of complementary DNA probes with target DNA was occurring, and not simply attachment of the probe to the chemically altered glass surface, the technique of surface plasmon resonance (SPR) imaging was used to measure DNA hybridization. SPR imaging is a surface-sensitive, optical detection method that can be used to quantitatively measure the amount of hybridization that occurs in an *in situ* environment, as the presence of DNA in solution does not interfere with the detection of hybridization at the surface (*Analytical Chemistry*, 69, 4939-4947, 1997). To use SPR techniques, DNA must be spotted on a noble metal surface (e.g., gold) (*J, Amer. Chem. Soc.*, 121, 8044-8051, 1999).

Target DNA on the ATMS-treated/diazotized gold surface was hybridized with a complementary DNA probe. The diazotized and spotted gold-coated slide was placed in a sealed cell with 2 ml 3X SSC and 20 µl 10% SDS. 300 ng of single-stranded labeled *am*-DNA and 0.6 µl 10% SDS was injected into the cell 11-12 minutes after the experiment began. The experiment proceeded for 72 hours at room temperature.

SPR spectroscopy was used to study the hybridization of the *am* DNA probe to the immobilized *am* DNA. FIG. 10 shows the time vs. reflectance line profiles collected during the course of the experiment as hybridization proceeded. An increase in reflectance was observed until about 7 hours after the onset of the

experiment, when a plateau level was reached. This profile reflects a thickening at the surface of the slide causing a shift in the reflection pattern. This shift is due to the complementary binding of probe to target DNA on the surface of the slide, with steady state hybridization occurring at about 7 hours.

Although the present invention has been fully described in conjunction with the preferred embodiment thereof with reference to the accompanying drawings, it is to be understood that various changes and modifications may be apparent to those skilled in the art. Such changes and modifications are to be understood as included within the scope of the present invention as defined by the appended claims, unless they depart therefrom.